Vol. 17 No. 9

November, 1951

THE BOTANICAL REVIEW

Interpreting Botanical Progress

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Published Monthly except August and September at
111 E. Chestnut St., Lancaster, Pa.

Entered as second-class mail matter February 4, 1935, at the Post Office at Lancaster, Pennsylvania, under the Act of March 3, 1879.

THE BOTANICAL REVIEW

Founded in 1935 by
HENRY ALLAN GLEASON and EDMUND H. FULLING
Managed, edited and published at
The New York Botanical Garden
by
EDMUND H. FULLING

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Articles are obtained primarily by invitation. Unsolicited manuscripts are also welcome. In all cases, however, the editor reserves the right to accept, reject or suggest revisions in submitted material. All manuscripts should be on double-space typewritten pages, with references and citations in accordance with the customary style of THE BOTANICAL REVIEW.

Missing numbers can not be supplied unless notice is received within two months after appearance of issues concerned.

All correspondence should be addressed to THE BOTANICAL REVIEW, at 111 E. Chestnut St., Lancaster, Pa., or Fordham P. O., New York, N. Y.

THE BOTANICAL REVIEW

VOL. XVII NOVEMBER, 1951 No. 9

THE CROWN-GALL PROBLEM

R. S. DE ROPP[1]

The New York Botanical Garden

INTRODUCTION

Since the discovery (141) of the bacterial etiology of crown-gall, this disease has been investigated by many students of pathological growth, partly, no doubt, because of the repeated assertions by Ervin F. Smith that crown-gall of plants is a cancer (128–130, 132). Since Smith and his colleagues carried out their pioneer researches, a great deal of new information about the crown-gall problem has become available. It is the purpose of this paper to review the earlier statements of Smith in the light of this recent work. The general description which Smith (130) gave of the disease can be used as a basis for further discussion of its various aspects. Crown-gall, he states, " is a peculiar hyperplasia, the bacteria which are the cause of it developing sparingly and only within the cells, which they compel to divide early and repeatedly so that a great mass of non-capsulated, small-celled tumor tissue arises in which the bacteria themselves are invisible. These numerous, small, incompletely developed, atypical, unripe cells are endowed with enormous reproductive capacity which is not under the physiological control of the plant and the continued development of which is detrimental to the plant both locally and constitutionally. The tumors, even when deep seated, are often incompletely vascularised, often quite fleshy and very subject to decay. These conditions naturally result in the production of open wounds, which are subject to a variety of secondary infections, some of them very harmful to the plant. The tumor when inoculated on fleshy roots such as the sugar beet is often larger than the root itself ".

" Physiologically and structurally the tumor resembles cancer in many ways. As ordinarily seen it certainly exhibits growth inde-

[1] Present address: Lederle Laboratories, Pearl River, N. Y.

pendent of function. Vegetative activity is stimulated, functional activity is depressed . . . . It can be stimulated by feeding or starved into quiescence similarly to mouse cancer. It can be grafted onto other plants of the same species. Like cancer it can be cut out but will return if all has not been removed. It often grows with astonishing rapidity and destroys tissues both by compression and by infiltration. Sometimes the tumor recedes of its own accord, but whatever immunity is worked up in the plant seems to be temporary. The diseased plant may also show something corresponding to cachexia in animals . . . . The cells of the tumor are often markedly embryonic in their character and show in different tumors, and also in different parts of the same tumor, various grades of undifferentiation, the more embryonic cells exhibiting a very strong affinity for stains . . .".

" As in cancer, the nuclei of this tumor divide both mitotically and amitotically. Nuclear division by simple cleavage is rather common. Variously lobed, notched and cleft nuclei are as common in crown gall as in animal neoplasms. Cells containing several nuclei are produced in this way and are common . . . . There is a marked disturbance of polarity in cells and vessels . . . . As there may be an inflammatory reaction around a cancer, so in the vicinity of a crown-gall there may be excessive nutrition and growth leading to enlargement of parts not actually parasitized . . .".

" Deep seated secondary tumors occur frequently often at a considerable distance from the primary tumor . . . . They contain the same intracellular organism as the primary tumors. So far as can be determined these secondary tumors are not produced by the mechanism which obtains in the case of the olive tubercle, caused by *Bacterium savastonoi,* or as human and animal tuberculosis. In both these diseases the secondary growths are caused by direct bacterial migration either internally through special vascular channels, or externally from ruptured diseased surfaces to fissured healthy surfaces, the bacteria in the new locations setting up local irritations that result in inflammatory (granulomatous) cell reactions repeating the old pattern of the disease. On the contrary, in crown gall the secondary tumors are outgrowths from a tumor strand, inasmuch as microscopic examination has shown that they are connected with the primary tumor by means of a chain of tumor cells ".

Smith distinguished crown-gall tumors, which he defined as "sarcomas" (connective tissue tumors), and others, which he defined as "carcinomas" or "epitheliomas". He also described embryonic teratomas in which the tumor tissue was intermingled with a mass of young roots and shoots. The type of tumor produced depended on the type of tissues inoculated. In the primary tumor of the Paris daisy, in addition to the tumor cells which could arise from the cortex and from the deeper true cambium, Smith observed a supporting stroma which he described as an ingrowth from the stem. This stroma consisted of both cells and vessels. It was Smith's opinion that the stimulus which causes the abnormal proliferation in crown-gall is a bacterial endotoxin produced by the intracellular parasites which acts on the cell nucleus of the host plant only when the bacteria are dead and their membranes have become permeable. These views of Smith regarding the nature of crown-gall will now be discussed in the light of evidence which has become available since his paper was written.

THE CAUSAL ORGANISM

In their original paper (141) Smith and Townsend proposed the name *Bacterium tumefaciens* for the crown-gall organism. It was described as motile, non-gas-forming and aerobic with all the sugars and alcohols tested (dextrose, saccharose, lactose, maltose, mannite and glycerin). The colonies were white on standard nutrient agar. In tubes of bouillon it grew best at the top, producing a stringy ragged rim easily separable on shaking. The surface colonies on agar were small, round, smooth and rather dense. It did not liquify gelatin and would not grow at blood heat. The organisms were described as medium sized short rods with rounded ends often in pairs with a plain constriction, the elements usually being one micron or less in diameter and two or three times as long. The one to three flagella were polar.

The organism was subsequently renamed *Pseudomonas tumefaciens, Phytomonas tumefaciens* and finally *Agrobacterium tumefaciens* (25). In Bergey's manual (2) the organism was characterized by its capacity to use inorganic forms of nitrogen and to produce nitrite from nitrate to a slight extent. It is a Gram-negative organism and is capsulated (51). In old cultures it forms a zoogleal mass in which the bacteria appear as faintly staining cocci. Other bodies seen in this zoogleal mass have been inter-

preted as spores (84). There is no other evidence, however, that this organism is a spore-former.

In the course of its life history the organism forms peculiar star-shaped aggregates of cells which occur with particular frequency when the organisms are grown on a medium containing dilute carrot juice and 0.01% each of $FeSO_4 \cdot 7\,H_2O$ and $MnSO_4 \cdot 7\,H_2O$ (145). The aggregates have been studied by means of the electron microscope and with the aid of the Feulgen stain (10, 143). In a small percentage of instances there was found what appeared to be a fusion of the Feulgen-positive material at the centre of the aggregates. It was concluded (10) that: "Although this finding is highly suggestive of a simple form of sexuality in these bacteria, it is nevertheless felt that cytological studies alone are inadequate to determine the answer to this question with certainty". Claims have been made that, at one stage of its life history, the crown-gall organism is capable of passing through a Berkefeld filter (123). This finding has not been confirmed.

The crown-gall organism is able to utilize ammonium nitrate as the sole source of nitrogen in the presence of glucose. Ammonia nitrogen was more available than nitrate nitrogen. The polypeptide and amino nitrogen of peptone was readily utilized by these organisms (26). It ferments glucose with production of CO_2 (27), and the rate of sugar fermentation is increased by addition of manganese to the medium. Iron and zinc are also of importance to the nutrition of the crown-gall organism. Its growth is only slightly increased by addition of yeast extract to the medium. The organism was shown to synthesize unusually large quantities of biotin, large amounts of riboflavin and moderate amounts of thiamine and pantothenic acid. It is also able to synthesize all other factors of this nature which it requires for its growth (105).

HOST-PARASITE RELATIONSHIP

Smith was of the opinion that the crown-gall organism is an intracellular parasite, and he claimed that, by the use of special techniques, the presence of the bacteria in the cells can be demonstrated (127). Subsequently, however, he admitted that he could not say unqualifiedly that he had ever seen the organism within the cell (2). A number of later studies supported Smith's opinion that the organism is intracellular (5, 100, 112, 113). Others, however, suggested that the crown-gall organism lives between

the cells of the host in the form of zoogloeal strands lying in the vessels or intercellular spaces (114, 122). Injection of living crown-gall organisms directly into the trichome hair cells and epidermal cells of tomato stems did not result in formation of galls, and the conclusion was drawn that " the living cell interior is an unfavorable medium for these bacteria " (56).

These studies are not conclusive. Although crown-gall bacteria have been demonstrated in the intercellular spaces of infected tissues, we can not say with any certainty that these were the organisms which brought about the transformation of the tissue from the healthy to the tumorous condition. The fact that injections of bacteria into tomato trichomes failed to give rise to tumors may show only that these specialized cells are not in a state to respond to the tumefacient stimulus. If the bacteria are capable of exerting their action through the cell wall, then it should be possible to obtain tumors by applying the organisms to uninjured tissue. It has been found, however, that galls are not formed under these conditions. Wounding of the tissue at the time the bacteria are introduced seems to be essential if tumor formation is to result (115). The number of bacteria introduced is not important, but the size of the wound directly affects the size of the gall (56, 84). Wounded tissue ceases to respond with gall formation to the application of crown-gall bacteria in from five to seven days (7, 12, 34, 115). This is about the time needed for the wounded tissue to become covered with a growth of fresh callus. It is possible that wounding of the tissues plays a role in tumor production because it supplies some stimulus such as a " Wundthormon " required for proliferation of the tissue. It seems more probable, however, that the role of wounding in tumor formation is to expose the protoplasts of a certain number of cells to direct action of the crown-gall bacteria. Possibly only those cells whose protoplasts are exposed in this way undergo the transformation from healthy to tumor cells. All the available evidence suggests that the factor in the crown-gall organism which causes tumorous transformation is unable to exert its effect through the intact cell wall.

THE TRANSFORMING PRINCIPLE

Smith's suggestion that the factor responsible for transforming healthy into tumorous cells is an endotoxin liberated on the death

of the bacteria has gained little support from more recent research. Many attempts have been made to induce gall formation in various susceptible plants by means of extracts of the crown-gall organism. A wilt-inducing polysaccharide has been isolated from the crown-gall organism (58), but, although more of this substance appears to be formed by virulent than by attenuated strains of the bacteria (57), there is no evidence that it is directly concerned with tumor formation. Other tests with fractions of the crown-gall organism (90) have shown that the phosphatide fraction produced cell proliferation, and the polysaccharide fraction produced necrosis with limited cellular proliferation.

The ability of the crown-gall organism to generate indole-3-acetic acid from tryptophane (1, 91) has suggested that the action of the bacteria on plant tissues is due to their generating this substance. Indole-3-acetic acid is capable, when applied in lanolin mixtures containing 1% to 3% of the active substance, of inducing formation of massive tumors on a number of plants. Filtrates and ether extracts (17, 96) of the crown-gall organism have also been found to possess tumefacient power.

There are certain differences between the tumors induced by the crown-gall organism and those produced by indole-3-acetic acid which make it seem highly unlikely that the bacteria exert their effect by generating this substance. It has been shown, however, that indole-acetic acid and a number of related substances (11, 117, 150) have the capacity to cause tumor growth on stems inoculated with attenuated strains of crown-gall bacteria which, without the growth substances, do not cause tumor formation. This finding has led Braun and Laskaris (11) to suggest that "there appear to be at least two distinct phases involved in tumor formation. In the first phase, the normal host cells are changed to tumor cells which without stimulation will not develop into a neoplastic growth. The second phase consists in the stimulation of the changed cells to continued multiplication by a growth substance, resulting ultimately in the formation of a tumor". They also state that the difference between attenuated and virulent strains of the crown-gall organism may lie in the relative amounts of growth substance produced by each, either as a product of bacterial metabolism or by the host under the influence of these organisms.

Transformation of healthy to tumor tissue by the crown-gall organism is strongly affected by the temperature at which the

tissue is held after inoculation. Early studies (116) revealed that the development of galls on tomato plants was slight between the temperatures of 28° and 30° C, and that above 30° C no tumors developed. Both the crown-gall organism and the tomato plants grew well at this temperature, but the process of tumor induction was inhibited. In certain species of *Nicotiana* galls were produced both above and below the critical temperature, but the tumors that developed at 31° C were smaller and made their appearance ten days later than did those produced at 26° C (120). In periwinkle the conversion of normal cells to tumor cells failed to occur when the inoculated area of the plant was maintained at a temperature of 32° C (7). Periwinkle plants maintained at 26° C for one, two, three, four and five days before being transferred to a temperature of 32° C differed significantly in the size of tumors they developed. Plants transferred after one day at 26° C failed to develop tumors; transferred after two days at this temperature they developed a few small tumors; after three days the tumors were larger; after four and five days the tumors attained the same size as those on plants maintained continuously at 26° C. The high temperature did not inhibit the growth either of the plant or of the bacteria, nor did it exert its effect by producing a physiological disturbance in the plant as a whole. There was apparently a lag of somewhat more than one day between infection and initiation of the healthy-to-tumor cell transformation.

Exposure of *Vinca rosea* plants inoculated with crown-gall bacteria to temperatures of 46° to 47° C caused destruction of the bacteria without killing the plants (7). By this means it was found that when the crown-gall organisms were eliminated from the plants after 36 to 48 hours, galls still developed at the sites of inoculation. The most significant aspect of this finding, that crown-gall tumors are capable of continuing to grow in the absence of the inciting agent, will be considered later. Here it is important to note that the time taken to bring about the transformation from healthy to tumor tissue is relatively short. Tumors formed on periwinkle plants which had been freed from bacteria after 36 hours remained very small. Apparently the bacteria had to act on the tissues about four days for the altered cells to receive a maximum of stimulation.

In *Kalanchoë* also the transformation of healthy to tumor tissue took place at 25° C but not at 32° C (12). The minimum period

needed for the bacteria to alter normal cells to neoplastic cells was 32 ± two hours at 25° C. When the plants were held for 24 hours at 32° C, then placed for ten hours at 25° C, the cellular transformation was still accomplished. Evidently the bacteria required a period of 20 to 24 hours at either 25° C or at 32° C for environmental adjustment plus an additional ten-hour period at 25° C to produce the transforming principle in the amounts necessary to bring about the cellular alteration. The relative size of crown-gall tumors apparently depended on the amount of the transforming principle available at the time of the cellular alteration.

These findings indicate that normal plant cells are converted to tumor cells by the crown-gall bacteria at 26° C but not above 30° C. Once the cellular transformation has been accomplished at the lower temperature, the tumor cells develop into a neoplastic growth equally well, both above and below the critical temperature. By utilizing this finding it is possible to stop the transformation process abruptly at any desired time simply by growing the plants at a temperature above 30° C. The amount of transformation that occurs at the lower temperature can be measured by the relative size of the tumors that develop at a temperature above 30° C.

The most recent studies on the transforming principle (9) indicate that for *Kalanchoë* the optimal temperature for transformation is from 25° to 27.4° C. Between 27.5° and 28.8° C there was progressive decrease in the size and weight of the resulting tumors and a delay in the inception period. Above 29° C tumors were not initiated. These results have been interpreted as indicating that the transforming principle is an exceedingly thermolabile agent which at 29° C is destroyed as rapidly as it is formed. "When the activation energy was computed according to Arrhenius equation

$$Ea = \frac{R\,T_1\,T_2}{T_2\,T_1} \ln \frac{K_2}{K_1}$$

from data obtained at 27.3° C and 28.6° C, very high values of more than 80,000 calories per mole were found. Since reactions of this order of magnitude are characteristic of protein denaturation, the results suggest that either the tumor inducting principle or something intimately associated with its inactivation is a factor of complex structure".

Virulent crown-gall bacteria can be maintained for as long as three months at a temperature of 33° C without losing their tumefacient power (34), which suggests that this temperature, although it apparently destroys the tumor-inducing principle, does not prevent its production. Grown on a medium containing 0.1% to 0.3% glycine and a number of other amino acids, virulent strains of the crown-gall organism have been found to lose their tumefacient power (99, 155). Restoration of the bacteria to a medium free of these amino acids was sometimes accompanied by a return of tumefacient power. Tumor formation by crown-gall bacteria was also prevented by a concentration of 0.5 mg./ml. of streptomycin (33). Lysis of crown-gall bacteria with bacteriophage destroyed their tumefacient power (61).

As the transforming principle of the crown-gall organism has not been isolated, both its nature and its mode of action remain matters for speculation. It might be a chemical or a physical agent. If chemical then it could be either a relatively simple carcinogenic agent like methylcholanthrene or a macromolecular entity. The weight of evidence favors the second hypothesis. Such a macromolecular entity might be a fraction of the bacterial cell itself, having the capacity to induce a self-perpetuating abnormal pattern of growth in the host cell. The transforming principle of the pneumococci (104), which is capable, in a concentration of one in 600 million, of permanently transforming R pneumococci into the encapsulated form, provides an example of a biologically produced transforming agent. The Kappa particle in *Paramecium* (142) appears to be a similar factor. The transforming principle might equally well be interpreted as a virus, of which the crown-gall organism is the vector or a normal constituent of the host cell which by action of the bacteria is converted into a tumor-inducing substance.

We have no way of knowing whether the change brought about by the transforming principle is confined to the cytoplasm or whether the nucleus is also affected, whether nuclear genes are involved or only plasma genes. As the formation of gametes does not appear to take place in tumor tissue, it is hard to see how this question can be answered. Probably the transforming principle can best be described as a mutagenic agent of considerable complexity, capable, under certain special conditions, of altering perma-

nently the growth pattern of cells of a large group of higher plants. Identification of the transforming principle is perhaps the most fundamental task of those engaged in the study of the crown-gall problem.

SPECIES AND TISSUE SUSCEPTIBILITY

Smith (125) observed that the crown-gall organism has the capacity to stimulate gall formation on plants belonging to 18 widely separated botanical families. Species in the following families have been found susceptible: Salicaceae, Juglandaceae, Fagaceae, Moraceae, Polygonaceae, Chenopodiaceae, Caryophyllaceae, Ranunculaceae, Lauraceae, Cruciferae, Resedaceae, Crassulaceae, Rosaceae, Leguminosae, Geranaceae, Tropaeolaceae, Rutaceae, Euphorbiaceae, Anacardiaceae, Celastraceae, Balsaminaceae, Vitaceae, Malvaceae, Sterculiaceae, Passifloraceae, Begoniaceae, Cactaceae, Myrtaceae, Oenotheraceae, Umbelliferae, Ericaceae, Ebenaceae, Oleaceae, Apocynaceae, Polemoniaceae, Labiatae, Solonaceae, Caprifoliaceae, Compositae (40).

The plants on which crown-gall tumors have been observed are almost all dicotyledons. Stapp (144), however, has reported tumors on asparagus. Experiments have been made to determine whether the crown-gall organism can cause tumor production in animals. Small nodular growths were obtained in brook trout inoculated in the eye socket with these organisms (130). The action of the bacteria on a number of invertebrates has been tested (65, 151–154). Tumors were produced on *Nereis diversicolor*. It has been claimed that at a distance the organism can stimulate multiplication of cells in the bone marrow of rabbit (147).

Crown-gall tumors have been described on almost all the organs of susceptible plants, including stems, leaves, roots, buds and storage organs. Smith (130) described many tumor types differing in structure according to the tissue from which they were derived. "Ordinary crown galls" he compared to sarcomata because they involved connective tissue and developed most vigorously on young plants, producing large perishable tumors, many of the vessels in which were reduced to their primitive elements and had very thin cell walls. He described tumors derived from epidermal, cortical or gland cells which he compared to carcinomata. Embryomata were also described in which the tumor tissue was mixed with organs, roots, buds, leaves or flower parts. Many of

these tumors developed shoots or leaves on their surfaces. He assumed that these organs were formed not from tumor cells but from normal cells which had been torn from their place in the course of tumor growth. Shallow inoculation of stems penetrating only into the outer cortex resulted in the formation of superficial hyperplasias, the growth of which was limited. These tumors involved neither wood, cambium nor phloem. They were formed solely from cells of the cortex which, under the influence of the bacteria, begin to grow rapidly, become smaller and more embryonic in their nature than the surrounding cells. They were able to vascularize themselves to a limited extent. Smith concluded that this proved that the action of the crown-gall organism resulted in the conversion of ripe cortical cells into a more embryonic tissue.

When the stems of tomato plants were stroked with a glass rod carrying crown-gall bacteria, exceeding small galls were produced which evidently developed from the hair cells of the epidermis (56). The smallness of these tumors suggests that galls produced from such cells either have a limited proliferative power or are inadequately vascularized. Anatomical studies on several species of plants inoculated in different tissues with crown-gall bacteria led to the conclusion (111) that participation of the cambium is not necessary for tumor formation. The pith, cortex and vascular parenchyma all proved capable of proliferating under the influence of the crown-gall bacteria. When cambial tissue was inoculated, the resulting tumors were larger than those which developed from non-cambial tissue; this, however, was not accepted as proof that the cambium plays a decisive role in tumor formation, but was interpreted as resulting from closer connection of the cambial tissue with the vascular apparatus of the plant.

Inoculation of isolated fragments from the storage organs of various plants has shown that cells of such tissues differ in their capacity to respond to the stimulus exerted by the crown-gall organism (39). Such tissue fragments, taken from the storage organs of carrot, beet and Jerusalem artichoke, developed tumors when inoculated with crown-gall bacteria which followed closely the lines of the cambium. In carrot the tissues of the phloem lying closest to the cambium also had some capacity to respond. Tissue of the secondary xylem was almost incapable of response. This difference in response was equally evident whether the tissue frag-

ments were supplied with nutrients in the medium or cultured on plain agar. When whole carrot roots were sectioned longitudinally or transversely and inoculated with crown-gall bacteria on the freshly cut surfaces, the same distribution of tumor formation was obtained. Crown-gall tumors on isolated fragments of sunflower stem cultured in vitro showed a similar distribution (34). It would appear from these findings that the more closely a cell approaches to the meristematic condition, the more readily is it transformed into a tumor cell by the crown-gall organism.

Callus formation on carrot fragments cultured in vitro takes place almost exclusively from the radical (lower) as opposed to the foliar (upper) side of the fragment. Tumor production on carrots inoculated with the crown-gall organism is uninfluenced by this polarity (39). The amount of tumor tissue generated on the radical side of such a fragment is as great as that produced on the foliar side. In this respect crown-gall tumor tissue on carrot differs from the overgrowths which can be induced by indole acetic acid, the growth of which is influenced by the polarity of the organs.

Considerable variation in reaction to the crown-gall organism has been found, even among plants of the same species (39, 101, 102). Carrot storage organs of the variety Chantenay could be divided into three groups—strong reactors, weak reactors and non-reactors—according to the amount of tumor tissue they generated when inoculated with crown-gall bacteria.

SECONDARY TUMORS

The frequent occurrence of secondary tumors at a distance from the primary ones was described by Smith (130). He declared that these tumors did not result from bacterial migration within the plant or on its external surface, but were the consequence of outgrowths from the primary tumors which, in the form of tumor strands, had grown through the healthy tissues. These strands of bacterially infected cells were sometimes large enough to be visible to the naked eye. More often they consisted of a delicate thread of cells and vessels sometimes hard to find even under the microscope. In tobacco such strands were found only in the outer cortex. In Paris daisy they grew through the region of the pith periphery. The secondary tumors reproduced the structure of the

primary tumors, in which respect they were thought to resemble the metastases in animal cancer.

In a subsequent paper (137) Smith stated that tumor growth in the plant could occur not only by tumor strands but also by apposition. Tumor growth of this kind resulted from the conversion of healthy cells immediately in contact with the tumor tissue into tumor cells. In the course of being thus converted the healthy cells first enlarged, then began to divide with great rapidity. This rapid cell division resulted in the production of cells indistinguishable from those in the body of the tumor. The stimulus causing this proliferation was conceived as "a chemical-physical one derived from the bacteria and acting either at a distance from them, i.e., on cells in which they are not present, or as due to a direct transfer of bacteria from cell to cell" through pits in the cell walls. The narrowness of the appositional layer (0.5 mm. or less) was thought to indicate that the spread of the tumor must be due to the direct movement of bacteria from cell to cell rather than to the action of chemical products at a distance.

Different interpretations of the mode of origin of secondary tumors were advanced by other workers. In some plants it was stated that secondary tumors are formed when immature tissues are infected and the infected cells are subsequently spread out by growth (115, 122, 151). In *Nicotiana affinis,* however, the bacteria were found to migrate in zoogleal masses for a distance of several inches, though the intercellular spaces and secondary tumors in this plant were thought to arise from cells lying in the immediate vicinity of these masses of bacteria (122). Many of the tumor strands described by Smith were interpreted as being internal tumors formed at points where vessels had ruptured (148).

Crown-gall bacteria are known to be capable of moving considerable distances within the vessels of infected plants, carried by the transpiration stream (148). They have been isolated from apparently healthy tissue at a distance of over 100 cm. above and below the primary tumor in *Datura tatula* (146), and from intact internodes of sunflower 5.5 cm. from the point of inoculation one hour after inoculation. The bacteria have been found capable of passing through isolated stem fragments nine mm. long in about four minutes (35). Secondary tumors and tumor strands may be

produced in sunflower by an attenuated strain of the crown-gall organism, even though no primary tumor is formed. It has been thought probable that the mechanism involved in the formation of the secondary tumors is essentially different from that concerned in the formation of the primary tumor. It has been suggested that the initiation of these structures is brought about by diffusible cell-stimulating substances formed by bacteria which remain confined to the vessels of the plant (5).

ISOLATION OF BACTERIA-FREE CROWN-GALL TISSUE

Smith frequently emphasized that the crown-gall organism is present in tumors only in small numbers, and in several instances he was unable to isolate the bacteria from what appeared to be typical crown-galls. Nevertheless he insisted in all his writings that the bacteria were intimately associated with the tumor cells which they parasitized and that they multiplied as the host cells multiplied. That this idea might not be correct was first indicated when presumed crown-gall tumors on cultivated varieties of *Beta vulgaris* were grafted to other varieties (60, 80). Such grafted tumors grew on their new hosts without becoming differentiated, thus forming new tumors corresponding entirely in appearance and structure to the mother-tumor. These tumors, in a large number of instances, were found free of crown-gall bacteria. From this it was concluded that " the cells of the tissue, under the influence of the bacterium, become for a series of cell generations altered, and develop the abnormally increased proliferative power of tumor cells proper, which is independent of continued stimulation " (60).

Later studies of the bacterial content of crown-gall tumors on sunflower showed that, whereas primary tumors were 100% infected, secondary tumors on the first node above the primary were 83% infected, and at higher sites were only 4% infected (14). From such secondary tumor tissue fragments were removed aseptically and placed on an agar medium containing 2% sucrose, mineral elements, glycine and thiamine (160). A high proportion of these tissue fragments proved free of the crown-gall organism, and a number of them began to grow on the medium. This growth resulted in the formation of white friable tissue masses which did not differentiate and showed no signs of any polarity in

their growth. Cultures of healthy sunflower tissues were also prepared. The growth of such tissue fragments showed marked polarity. They often differentiated well organized vascular elements and became fibrous and woody. This tendency to a high degree of differentiation was accompanied by the formation of large amounts of chlorophyll and frequent formation of roots. Tissues were also removed from tumors induced chemically by means of indole oxalo acetic acid. The unilateral, compact, green, woody and sometimes rooted type of development was also characteristic of tissues taken from these tumors. Cultures of healthy tissue increased about 250 fold in a year's time, those of tissues from indole oxalo acetic acid tumors increased only about 50 times in the same period. Growth of the crown-gall tumor tissue was so rapid that its increase during 30 passages was estimated at one hundred million million times. Some of the strains of tumor tissue isolated at that time are still growing (1950), demonstrating that their capacity for growth on the medium used is unlimited.

Four hundred cultures of this crown-gall tumor tissue were crushed individually in nutrient broth and incubated from one to eight hours. They were then mixed with nutrient agar and poured into Petri dishes. Most of these plates proved free of bacteria. The bacterial colonies which did appear were tested by inoculation into tomato. None of them proved cabable of producing galls. Serological tests designed to detect the presence of the crown-gall organism were uniformly negative. It was therefore concluded that the cultures of crown-gall tissue isolated from these galls were entirely free of the crown-gall bacteria.

On the assumption that the difference in behavior between bacteria-free tumor tissue and healthy tissue might have been due to a virus, 100 cultures of tumor tissue were ground on sterile glass slides, and the debris of each batch inserted into a one-month-old sunflower plant at the cambial level. Only one tumor developed on these plants. It proved to be bacteria-free and was attributed to a few cells of the culture which remained uninjured by the grinding. In a second experiment no tumors were obtained. This indicated that, if the abnormal proliferative activity of the tissues was due to a virus, this could not be of the stable easily transmissible sort exemplified in the tobacco mosaic group and in the chicken sarcoma I. "While this does not preclude the possi-

bility of a virus of different behavior having been involved, there is no evidence to suggest the existence of such a virus, and it would surely be superfluous to postulate the existence of such a factor in the absence of any evidence".

When bacteria-free crown-gall tissue was grafted back onto sunflower plants, a tumor resulted whenever the graft took. Similar results were obtained when the tissue was grafted to the stem of artichoke (*Helianthus tuberosus*). These tumors apparently arose largely, if not entirely, from preexistent tumor cells. There was no evidence that the host cells were themselves modified in their behavior by the presence of the adjacent tumor cells.

Bacteria-free crown-gall tumor tissue was later also isolated from galls on *Vinca rosea* which had been freed of bacteria by heat (158). The tissue grew more rapidly than did that isolated from secondary galls on *Helianthus annuus*, was white in color and open in texture, and proved capable of indefinite growth on the same medium as had supported the growth of the sunflower tumor tissue. After 20 passages, fragments of these cultures were grafted into young healthy plants of *Vinca rosea*. In 33 out of 42 of these grafts tumors resulted closely resembling the tumors produced by inoculation of crown-gall bacteria. These tumors were interpreted as arising from the implant and not from the host cells.

Portions of primary galls on sunflower were also found in some instances to be free of bacteria, and from such galls also several clones of bacteria-free crown-gall tumor tissue were isolated. These clones differed from one another in growth rate and appearance, some being composed chiefly of soft parenchymatous cells, others mainly of tracheids (29). Further studies resulted in the isolation of bacteria-free crown-gall tissue from several other plants, including *Scorzonera, Helianthus tuberosus* (44, 46), Paris daisy, marigold (55), *Vitis vinifera, Opuntia monacantha, Carthamnus tinctoria, Abutilon avicennae, Nicotianum tabacum, Antirrhinum majus* (109).

PHYSIOLOGICAL CHARACTERISTICS OF CROWN-GALL TUMOR TISSUE

That the nutritional requirements of crown-gall tumor tissue differ from those of the stem tissues from which they arise was clearly demonstrated by the work of White and Braun (160).

Healthy stem tissue of sunflower was found capable of making some growth on the medium which indefinitely supported the growth of bacteria-free crown-gall tumor tissue. The growth made by such tissue, however, was small in amount and soon ceased entirely. When small fragments of sunflower stem tissue were cultured on this same medium in the presence of indole acetic, indole butyric or napthalene acetic acid, their growth pattern was strongly modified (30). On a medium containing 0.01 mg./liter of these substances, the stem fragments developed adventitious roots, the growth of which increased the weight of the fragment more than 50 times. On a medium containing ten mg./liter of these substances, isolated fragments of sunflower stem proliferated to give rise to a loose translucent tissue composed of thin-walled elongated cells. Fragments isolated from the cambium of *Vinca rosea* behaved in a similar fashion. Bacteria-free crown-gall tissue from these plants was grown on a medium containing the same concentrations of these substances. Its growth was not affected in any way by the lower concentration but was strongly inhibited by higher concentration. The substances had no noticeable effect either on the microscopic or on the gross structure of the tumor tissue. A similar study conducted with bacteria-free crown-gall tissue derived from *Helianthus tuberosus* (44), *Vitis vinifera, Abutilon avicennae* and *Nicotiana tabacum* (109) revealed that these tissues were also capable of growing indefinitely on a medium devoid of indole acetic acid, although tissue derived from the healthy plant requires this substance for its continued growth. Growth of crown-gall tissue of *Nicotiana tabacum,* however, was simulated by a concentration of 0.1 to 1.0 mg./liter of indole acetic acid (109).

The action of cysteine hydrochloride and nine other growth regulators on the growth of bacteria-free crown-gall tissue of sunflower has been tested (54). A slight increase in fresh weight was observed in the presence of very low concentrations of some of these substances. At high concentrations they all inhibited growth. Better growth of sunflower tumor tissue was obtained with some modifications in the original medium used by White and Braun, including the addition of 0.8 mg./liter pyridoxine (53). Optional temperature for growth of this tissue was 24°–28° C, optimal pH 5 to 6.4, optimal sucrose concentration 1%

(52). The effect of various carbon compounds on the growth of bacteria-free crown-gall tissue of sunflower, periwinkle, Paris daisy and marigold was tested (55). Dextrose, levulose and sucrose proved the best carbon sources for growth. Some growth was obtained on media containing polysaccharides and a number of other sugars. Organic acids and alcohols either inhibited growth or supported it very poorly. Excellent growth was obtained on media containing from 0.008 M to 0.064 M nitrate or urea (119), poor or no growth with nitrite, 11 amino acids permitted no growth. D,L-alanine enhanced growth at a 0.0001 M concentration, inhibited it at 0.002 M and enhanced it again at 0.128 M. None of the substances tested in the course of these studies brought about any marked increase in the growth rate of the tumor tissue. The original medium of White and Braun appears to provide all the material essential for the growth of crown-gall tissue. It is probable, however, that the growth rate of crown-gall tissues on this medium is below the maximum possible for this tissue, owing to the absence from the medium of certain as yet unidentified nutrients. Thus the growth of crown-gall tissue of *Scorzonera* has been more than doubled by the addition to the medium of 50% coconut milk (39*a*).

Crown galls when induced on storage organs of carrot or potato show a remarkable ability to utilize the food materials stored in these organs. Crown-gall tumors on the storage organ of carrot were removed and regenerated five times in succession and transformed about 15% of the estimated dry weight of the storage organ into tumor tissue in the course of 12 weeks. Fragments of potato tissue on which galls were induced were found to be completely devoid of starch after the gall had attained its full size. The stored materials in Jerusalem artichoke tubers bearing crown-galls were also almost completely utilized for the growth of tumor tissue (39).

The respiratory behavior of bacteria-free crown-gall sunflower tissue has been compared with that of healthy vegetative growing points, inflorescences and internodes of this plant (159). No qualitative difference in the respiratory picture of healthy and tumor tissue was found. The respiratory level of the tumor tissue was lower than that of the healthy tissue.

These nutritional studies indicate that bacteria-free crown-gall

tumor tissue possesses simpler nutrient requirements than do the tissues from which it is derived. Three explanations for this difference can be offered:

1. Tumor tissue possesses a nearer complete enzyme system than the tissue from which it is derived.

2. The process of tumefaction eliminates a growth inhibitor which limits the growth of healthy tissue.

3. The number of essential metabolites involved in the growth of tumor tissue may be smaller than that involved in the growth of healthy tissue. The tumor tissue is therefore less exacting in its nutrient requirements.

In the light of the available evidence the first of these hypotheses seems most probable. Crown-gall tumors produce, on the plants on which they develop, many of the symptoms, such as epinasty of petioles and inhibition of lateral buds, which are also produced by such growth hormones as indole acetic acid (19, 28, 97, 118, 124, 138). Several investigators have described "hyper-auxiny" of such tumors (92, 93, 94, 95, 98). Estimations of auxin content of healthy and crown-gall tumor tissue of *Helianthus tuberosus* have been carried out by means of the Avena test (67). The concentrations of auxin in healthy tissue was estimated on the basis of fresh weight at 0.3×10^{-8}, that of vigorously growing crown-gall tissue at 5×10^{-8}. Concentrations of auxin in healthy and crown-gall tissue of *Scorzonera* were almost identical with the above (68). Therefore it seems possible that the enhanced growth capacity of crown-gall tumor tissue as compared with normal tissue may depend on its ability to manufacture a growth hormone which may be either indole acetic acid or some kindred substance. Gautheret (45), discussing the relationship of crown-gall and cancer, favors this hypothesis. "It is certain", he states, "that the tumor cell differs from the normal cell in being able to elaborate for itself the auxins necessary for its proliferation . . . . One can, in fact, admit that the crown-gall organism produces a transformation in the cell which involves either the acquisition or the exaltation of a capacity to synthesize auxin; owing to their elaboration of this factor, essential for cellular proliferation, the tumorous elements multiply without limit thus giving rise to a neoplasm". Braun (6) speculates similarly: "The results can perhaps be interpreted equally well by assuming that the tumor

cells themselves have acquired, as a result of their transformation, a capacity for synthesizing large amounts of a substance which is probably similar to, but not identical with synthetic plant hormones. The continued production of this substance by the tumor cells could account for the continued unregulated proliferation of the affected cells ".

TUMOR INDUCTION BY BACTERIA-FREE CROWN-GALL TISSUE

The tumors which were obtained by grafting bacteria-free crown-gall tissue of sunflower or periwinkle onto healthy stems of these plants were interpreted as arising from the implanted tumor tissue (158, 160). No evidence was seen in such grafts to suggest that healthy tissues adjacent to the tumor were converted into tumor tissues. Further studies of the interaction of healthy and normal tissues were made by means of in vitro grafts in which small segments of stem or fragments of healthy cambial tissue were used as the stock, fragments of tumor tissue being used as scion (28). In such grafts growth of the healthy scion was stimulated in two ways. Sometimes the scion reacted with the production of a mass of hyperhydric translucent tissues and adventitious roots. This reaction, so similar to that stimulated by certain concentrations of indole acetic acid, was thought to result from the production of this or a similar growth substance by the tumor tissue. Other scions which became firmly united with the tumor tissue failed to show this reaction but developed instead spherical tumors which equalled or exceeded in size that of the original fragment of healthy tissue.

All the tumors which developed in this way possessed a characteristic internal structure. The core of the spherical mass was composed of xylem in which short tracheids were mixed with parenchymatous cells. Outside the xylem lay a cambium, and outside this layer phloem tissue could be found which was devoid of sieve tubes. The outermost tissue of the tumor was a loose-celled cortex, the cells of which contained chloroplasts. These induced tumors differed in their structure from the tumor scion, within which a more or less chaotic mixture of cell types was found.

When fragments of these spherical tumors were removed and placed on White's medium they proved capable of growing at a

rate comparable to that of most strains of bacteria-free crown-gall tumor tissue. For several transfers they grew in the form of small spheres, each repeating the structure of the original tumor. After about six months in culture they began to lose their spherical form and their green color. Their structure became increasingly chaotic, though they still showed certain distinctive features which distinguished them from the original tumor scion.

Most of these spherical tumors arose apparently from the cambium of the healthy tissue in the immediate vicinity of the tumor graft. In one instance, however, a particularly massive tumor arose from the end of the stem fragment opposite the graft. Different strains of tumor tissue differed in their ability to produce these induced tumors. Strain K I, which was itself derived from an induced tumor, possessed the highest tumor-inducing power, giving 46 tumors in 48 graft unions (31). Wounding and treatment with indole acetic acid failed to bring about tumor formation on other parts of the stem to which the tumor tissue had been grafted.

Various extracts of tumor tissue were applied to fragments of sunflower stem to determine whether they possessed the capacity to induce tumor formation or cell proliferation (32). They proved incapable of producing either phenomenon. When the tumor scion was separated from the healthy stock by a thin layer of agar, it exerted no growth-promoting action on the healthy tissue. Only when a union occurred between tumor stock and healthy scion was any unusual growth observed in the scion. It was concluded that two different growth stimulants are produced by bacteria-free crown-gall tissue, one capable of causing generalized proliferation of tissues similar to that caused by indole acetic acid, the other capable of causing the formation of new tumors having a definite structure and the same nutrient requirements as the original tumor tissue. It seemed that both of these factors were capable of exerting their effects only when tumor and healthy tissue were united by a graft union.

Subsequent work in France (24) indicated that bacteria-free crown-gall tissue of *Helianthus tuberosus* will also, when grafted to healthy tissues of this plant, cause the production of outgrowths capable of growing on the same medium as will support the growth of crown-gall tumor tissue but not of healthy tissue. It is possible

to interpret these induced tumors as metastases of the original tumor scion, but there are several facts which make this explanation seem improbable. First, plant tissues are rigid, and migration of tumor cells through a stem seems improbable. Second, the induced tumors have, in all examples so far examined, a very characteristic structure which distinguishes them from the tumor scion. If these induced tumors are not metastases we must conclude that a transforming principle exists in the tumor tissue itself capable of changing healthy into tumor tissue. This transforming principle is apparently capable of passing only through a continuous layer of living tissue; nor does it seem able to travel far from the point of union of healthy stock and tumor scion. Possibly the original transforming principle has been transferred to and perpetuated in the tumor cells, or possibly some new transforming principle has developed in these tissues. Further research is needed to clarify this problem.

CHEMICAL TRANSFORMATION OF HEALTHY PLANT TISSUES

Smith (134) applied various chemicals in solution to the tissues of a number of plants, and succeeded by this means in producing tumors resembling in many ways those caused by the crown-gall organism. He was particularly impressed by the capacity of dilute ammonia to cause cell proliferation and stated: "I think I have established my original hypothesis—viz., that dilute ammonia causes intumescences and have rendered it probable that ammonia liberated within the cell in small quantities by the imprisoned bacteria must be one of the causes of the excessive and abnormal cell proliferation in crown-gall".

The fact that crown-gall tumor tissue continues to proliferate when freed from bacteria indicates that this theory is incorrect. Many other attempts have been made, however, to bring about a change in plant tissues by chemical means similar to that produced by the crown-gall organism. The discovery that several chemical substances are capable of causing cancers in animals stimulated this research, and several of these carcinogenic agents have been tested on plants. Immersion of seedlings of *Vicia faba* and *Pisum sativa* in a solution of coal tar for a short period caused vacuolization of the cytoplasm and nuclei and giant cell formation (63). Painting of various plants with solutions of Scharlach red, coal

tar and 1,2,5,6-dibenzanthracene resulted in the proliferation of tissues about the painted area which gave rise to small irregular masses of new tissue. The tumors induced by these means were all smaller than those produced on control plants inoculated with crown-gall bacteria (83, 86, 88). 3,4-benzpyrene and methylcholanthrene induced necrosis of the treated zone (87). Tissues of sunflower, various species of tobacco and of carrot were cultured in vitro in the presence of several concentrations of the carcinogens 1,2,5,6-dibenzanthracene, 3,4-benzpyrene and 20-methylcholanthrene. The conclusion was reached that the structural changes induced by the carcinogens in the in vitro cultures of carrot meristem are not malignant but are not unlike the plant tumors produced by the crown-gall organism (89, 89*a*, 89*b*, 89*c*).

Application of mixtures of indole-3-acetic acid and lanolin to bean plants resulted in the production of large galls on both the stems and the pods of these plants (66, 50). Similar reactions were induced by this agent on tomato plants (43). Structurally the tumors obtained by this means differed from those generated by the crown-gall organism in containing, in most instances, the rudiments of adventitious roots. Tumors produced by indole acetic acid were compared with crown-gall tumors by Levine (87) who stated: " The fundamental difference between the heteroauxin induced overgrowth and crown gall is the limited proliferation power of the former. The chemically stimulated cells perpetuate themselves for only a few generations ".

Sunflower stem fragments when grown in vitro in the presence of ten mg./liter indole acetic acid developed a mass of hyperhydric tissue which caused the fragments externally to resemble crown-gall tumor tissue (30). These fragments when transferred to a medium devoid of indole acetic acid differentiated into roots. It was concluded that treatment of tissue with indole acetic acid will not bring about any permanent transformation of the growth pattern of the tissue comparable to that produced by the crown-gall organism.

It has been shown, however, that carrot tissue maintained for several years on a medium containing 0.1 mg./liter indole acetic acid may undergo a change in many ways similar to that produced by crown-gall bacteria (41). This change was called by Gautheret " accoutumance ", a word which has been translated as " habitua-

tion". It involved a change both in the appearance and in the nutritional requirements of the carrot tissue. Cultures of habituated tissue were translucent and friable, whereas cultures of tissue which had not undergone this change were compact and opaque (109). The habituated tissue was capable of growth on a medium devoid of indole acetic acid, and its growth rate was not increased by addition of this substance to the nutrient. Cultures of the unchanged tissues grew very slowly in the absence of this substance and responded to its presence with considerable increase in growth. The change was evidently partially reversible for a strain of carrot tissue which had lost its sensitivity to indole acetic acid and became partially sensitive to this substance five years later, though it did not revert to its original condition.

Further studies revealed that this habituation is not confined to carrot tissue. It occurred also in tissue cultures of *Scorzonera* (43), Virginia creeper and *Vitis vinifera* (107). The culture of Virginia creeper (*Parthenocissus tricuspidata*) in which this transformation occurred had been cultured in the presence of napthalene acetic acid (0.3 mg./liter) for 14 months, after which it was transferred to a medium devoid of this substance. Growth was completely arrested in all but one of the six cultures. On a very limited area of this tissue a callus grew capable of developing in the absence of napthalene acetic acid. The rest of the tissue remained unchanged. The transformation in this instance appeared to be permanent (108).

The conditions under which this change occurred make it seem possible that "habituation" does not, as the name suggests, result from a tissue gradually acquiring the capacity to synthesize a growth factor after a period of contact with the substance. It seems more probable that the change was sudden and resulted from a somatic mutation within the tissue. The rarity of the transformation supports this view.

The isolation of bacteria-free crown-gall tissue from tumors on *Scorzonera* enabled Gautheret (47) to compare the behavior in vitro of normal, habituated and crown-gall tissue. Both habituated and crown-gall tissue proved capable of growing on a medium lacking indole acetic acid, although this substance was necessary for the optimal growth of normal tissue. The tumor tissue of *Scorzonera* had morphological characteristics similar to those of

the habituated tissue, though it was somewhat more friable. When cultured in the presence of different concentrations of indole acetic acid, the growth of habituated and crown-gall tissue was not significantly affected by concentrations of this substance ranging from 0 to 10 mg./liter but was inhibited by higher concentrations. The growth of normal tissue, however, was progressively increased by increasing concentrations of this material (46).

Fragments of habituated tissue of *Scorzonera* were grafted in vitro to healthy root tissue of this plant (22), and their behavior was compared with that of fragments of normal and crown-gall tissue similarly grafted. The fragment of normal tissue proliferated little and did not induce the production of a callus on the tissue of the stock in the vicinity of the graft. The fragments of crown-gall tissue proliferated freely and induced the production of a considerable callus on the tissue of the stock. Fragments of habituated also induced the production of callus on the normal stock, but this callus was less massive than that induced by the crown-gall tissue. Tissue was removed from the callus induced by the crown-gall tissue and by the habituated tissue (23), and cultured in isolation. The callus induced by the habituated tissue had a greater capacity to grow without indole acetic acid than had the tissue from which it had been derived and was less compact and opaque in appearance. It also had a smaller capacity to produce buds.

Normal, habituated and crown-gall tissue of *Vitis vinifera* were grafted into intact plants of the same species (13). Both normal and habituated tissues responded to the morphogenic influences of the plant and were incorporated into the stem without tumor formation. The crown-gall tumor tissue was uninfluenced by these morphogenic forces and grew as a spherical mass of tumor tissue similar in appearance to tumors induced by the crown-gall organism. It was concluded that "although the growth of habituated and crown-gall tumor tissue in vitro is similar, there is, in this instance, a pronounced and fundamental difference in their response when grafted into a healthy grape plant".

In their earlier papers Gautheret and his co-workers were inclined to interpret the "phenomenon of habituation to heteroauxin" as a "genuine tumorous transformation obtained by chemical means" (21, 109). Later they stated their opinion that

" tissues habituated to hetero-auxin possess properties intermediary between normal tissues and crown gall tissues " (22).

It is difficult to determine at the moment to what extent either of these views is correct. Is habituation a " tumorous transformation "? The grafting experiments of Braun and Morel suggest that the habituated vine tissue they used had not undergone a " tumorous transformation ". It did not, as did crown-gall tissue, form a tumor when grafted into the stem of an intact plant. Is habituation a transformation produced by chemical means, i.e., by the action of indole acetic or napthalene acetic acid? This question can also not be answered definitely. In tissues of Virginia creeper the transformation appeared 14 months after the tissues had been removed from the medium containing napthalene acetic acid. It is therefore possible that this chemical played no role in bringing about the change. Two characteristics distinguish habituated tissue from normal tissue, the friable translucent appearance of the tissues and their capacity to grow on media lacking indole or napthalene acetic acids. But the essential characteristic of bacteria-free crown-gall tumor tissue is not its appearance in vitro or its nutritional requirements but the fact that, when grafted back onto the normal stem, its growth does not conform to the growth pattern of the stem but results in the production of a spherical tumor. In fact, crown-gall tissue has lost its capacity for polarized growth. Habituated tissue still seems to possess this capacity. It is possible, however, that habituation represents one step in the direction of the tumorous condition, something equivalent to that " precancerous change " familiar to students of animal cancer. Different degrees of habituation may exist, and in its extreme form the transformation may be equivalent to that induced by the crown-gall organism. A great deal more work is needed before we can say with any certainty what this transformation really involves.

REVERSION OF TUMOR TO NORMAL TISSUE

Both Smith and Magnus (103, 130, 131, 135, 136) described tumors on a number of plants on which or within which organs existed. Some of these tumors contained roots in their interiors. Some bore small leaves or shoots on their surfaces. Smith concluded that these organs arose not from the tumor cells but from

the totipotent cells present in the tumor which had been stimulated to grow and differentiate. He stated: "The tumor cell is a disoriented degenerate cell, given over to hasty vegetative growth. It is not an embryo cell, and I know of no evidence going to show that it can develop subsequently into normal tissues, organs or the whole plant; on the contrary it tends steadily toward decay". Other workers disputed this view and claimed that leafy shoots may result from differentiation of crown-gall tissue (69, 72, 74, 75). In one clone of tumor tissue isolated from a primary crown-gall tumor on sunflower, roots were observed during the first few passages after its isolation. After more prolonged culture these structures vanished (29). Although many strains of bacteria-free crown-gall tumor tissue have been maintained in vitro, for several years no reports seem as yet to have been published describing differentiation of such tissues into any organs found on the normal plant.

Recent studies (8) have shown that strains of the crown-gall organism differ in their capacity to produce teratomas on *Kalanchoë daigremontana*. The T 37 walnut strain of the organism produced a particularly high proportion of complex tumors which showed varying degrees of morphological differentiation. The morphologically complex structures which arose on these tumors consisted mostly of leaves and stems. A few of these crown-gall tumors were composed almost entirely of highly distorted root-like structures. Decapitated plants were inoculated, and it was observed that the first response to inoculation was the formation of a typical crown-gall tumor. The morphologically complex structures arose out of this tumor. They were inoculated with crown-gall bacteria but failed, in most instances, to generate tumors. Plantlets which originated from leaves on teratomas produced sterile gall-like overgrowths in the place of roots.

Discussing these findings, Braun states: "The results described here can perhaps be interpreted by assuming the presence in plant cells of factors that determine the morphogenetic fate of the cells. In those instances in which undifferentiated tumors develop as a result of bacterial inoculation the factors concerned with morphogenetic determination must be assumed to be completely overwhelmed by the action of the tumor-inducing principle. The latter appears capable of cancelling in large part the various competences

for differentiation previously possessed by the affected cells. As evidence of this tissue cultures of tumors isolated from sunflower and *Vinca rosea* have now been kept in the laboratory for more than five years without showing any evidence of a tendency to differentiate and produce complex tissues and organs. When, on the other hand, this same tumor-inducing principle acts on young cells of plants such as *Kalanchoë*, the balance must be assumed to be in favor in varying degrees of the morphogenic determinants present in the affected cells. The result of such a condition would probably be the development of structural elements that show varying degrees of differentiation such as have been reported here. If this interpretation is correct, then it is of interest to note that the potentialities for differentiation in certain young cells appears to be so great that complete recovery of affected cells takes place and apparently normal plants are produced".

Subsequent work (8*a*) indicated that tumors arising on *Nicotiana tabacum* as a result of inoculation with the T 37 strain of the crown-gall organism can be induced to form normal shoots, to flower and to set seed. This complete reversion of crown-gall tumor tissue to a normal pattern of growth took place when all normally produced axillary buds were removed from tobacco plants that had been inoculated at the cut stem surface with crown-gall bacteria. This resulted in the production of a complex tumor that contained adventitious buds composed of tumor cells. When these buds produced shoots large enough to be used as scions, the shoots were removed and grafted to healthy tobacco plants from which again all normal buds had been removed. The tips of the shoots were again removed and grafted to healthy plants. After this treatment the stems, derived initially from tumor tissue, appeared to have reverted completely to the normal pattern of growth.

HISTOLOGY AND CYTOLOGY OF CROWN-GALL TISSUE

Smith (130) stated that, in crown galls, nuclei divide both mitotically and amitotically. Nuclear division by simple cleavage is common. Cells often contain several nuclei, and these nuclei may be variously lobed, notched and cleft. In later studies (78, 79, 81) no examples of atypical divisions were reported. Bi- and multinucleate cells were observed. Most tumor cells contained the diploid number of chromosomes. A comparison of the structure

of bacteria-free crown-gall tissue of artichoke with that of normal tissue, both cultured in vitro (42), showed that the structure of normal tissue was more orderly than that of the tumor tissue. In the normal tissue lignified elements were found associated with an island of phloem in which normal sieve tubes with sieve plates were visible. In the tumor tissue the lignified elements were not associated with islands of phloem, and sieve tubes were found to be entirely absent from this material. The lignified elements in bacteria-free crown-gall tissues occurred for the most part in isolated masses (6) and consisted of modified scalariform cells. Most of the tissue was composed of thin-walled parenchymatous cells. Giant cells were present which contained as many as 20 nuclei. Notched, cleft and lobulate nuclei were common in this tissue. The only abnormality observed in the mitotic picture was the occasional lagging of a chromosome.

CURE OF CROWN-GALL

A number of attempts have been made to bring about the recession of crown-galls by means of chemotherapeutic agents. Both penicillin and streptomycin have been described as having the power to destroy gall cells without injuring normal cells (15, 16, 49). Streptomycin was reported as being somewhat more efficient than penicillin. Studies of the action of these two substances on bacteria-free crown-gall tissue in vitro showed that penicillin G had an inhibiting action on the growth of this tissue at a concentration of 500 μg./ml. Streptomycin inhibited growth at a concentration of 50 μg./ml. (36).

Pteroylglutamic acid and 12 analogues of this substance have been applied to tumors induced on carrot by the crown-gall organism. Five of the analogues tested inhibited tumor growth completely when 0.05 μg. was applied per fragment. Even a dose of 0.0005 μg. per fragment significantly reduced the amount of tumor growth. The active compounds were 4-aminopteroylglutamic acid, 4-amino-N^{10}-methyl P.G.A., 4-amino-9, N^{10}-dimethyl P.G.A., 4-amino-9-methyl P.G.A., 4-aminopteroyl-γ-glutamyl-γ-glutamyl glutamic acid (37). 4-aminopteroylaspartic acid was later also found to have tumor-inhibiting activity. The materials were tested in vitro both on crown-gall tumor tissue of sunflower and on excised tomato roots and sunflower embryos. They dif-

fered in their activity. The compounds containing the methyl group as well as the 4-amino group partially inhibited the growth of tumor tissue at a concentration of one μg. per liter, the other compounds inhibited growth at ten μg./liter. The growth of the excised tomato roots was inhibited even more strongly than was that of the tumor tissue. Growth of excised sunflower embryos was also strongly inhibited. The inhibition was not prevented by any amount of pteroylglutamic acid. It appears that these compounds do not have a specific inhibitory action on the growth of tumor tissue. Cytological studies suggest that they act by preventing cell multiplication rather than cell enlargement. Onion roots grown in a solution of 0.1 mg./liter 4-amino-N^{10}-methyl P.G.A. were found to be completely devoid of mitoses after 24 hours (38), though mitosis again became evident after 48 hours.

Development of crown-gall tumors on the hypocotyl of sunflower could be prevented by local application of a solution containing ten mg./liter 4-amino-N^{10}-methyl P.G.A. This treatment did not have a harmful effect on the plant if the galls were treated after the hypocotyl was fully grown. When young stems were treated with this material the galls were prevented from growing, but severe damage was inflicted on the stem, resulting in the death or stunting of the plant (38).

Several nitrogen mustards, cortisone and guanazolo (8 aza guanine) also had an inhibitory action on the growth of crown-gall tissue in vitro. Most active of the nitrogen mustards tested was methyl-bis-β-chloroethyl amine. Exposure of tumor tissue for one hour to a concentration of 100 mg./liter of this substance completely inhibited its growth. A similar exposure to ten mg./liter reduced growth to half that of the controls. The growth-inhibiting action of this substance on excised tomato roots was stronger than its action on tumor tissue. Guanazolo inhibited the growth of tumor tissue in vitro at a concentration of one mg./liter. Cortisone was active at 100 mg./liter (38). Radium emanations have been found capable of killing crown-gall tissue, but the healthy tissue seemed to be equally sensitive to this agent (70, 76).

CROWN-GALL AND CANCER

Smith repeatedly emphasized the resemblance of crown-gall in plants to cancer in animals and was prepared to use the word

" cancer " to cover both diseases. " No broadly inclusive definition of cancer can be drawn that will not, along with the human and animal neoplasms, include also these plant tumors as true cancers " (130). He argued further that as cancer occurs in plants and is due to a parasite, cancer in animals must also be due to a parasite (126, 129). Many attempts were made to prove that bacteria were involved in the etiology of cancer, but none gave unquestionable positive results. The only parasites which have been conclusively shown to be concerned with cancer production are viruses such as the Rous sarcoma virus and the milk factor of Bittner.

Levine used the term " plant cancer " in the titles of a series of papers on crown-gall (72–74) but made it clear in several papers that he did not consider plant cancer to be analogous to animal cancer (69, 82, 85). The assertions of Smith that because crown-gall is caused by a schyzomycete the same must be true of animal cancer have been criticized on the grounds that bacteria isolated from cancerous tissues have never conclusively been shown to be causally related to the disease (121).

The discovery that crown-gall tissue freed of bacteria retains the properties of tumor tissue gave a fresh impetus to this discussion of the relationship of crown-gall to cancer. White and Braun pointed out (160) that the Webster's Unabridged Dictionary defines " cancer ", in the sense that it is universally used by laymen, as " any malignant tumor ". It defines " tumor " as " any abnormal mass of tissue, not inflammatory and independent in character, arising without obvious cause from cells of preexistent tissue, possessing no physiologic function and characteristically unrestrained in growth and structure ". " Malignant " is defined as " tending or threatening to produce death ".

Combining these definitions White and Braun have discussed their applicability to crown-gall tumor tissue. They point out that in drawing analogies between crown-gall and animal cancer one must bear in mind the great structural differences between plants and animals. The animal cell, for example, is characteristically mobile. It is not rigidly fixed to its neighbor cells and possesses the capacity to migrate, particularly along such channels as the lymph nodes. The plant cell, surrounded by a rigid cellulose wall, has no migratory capacity. In almost all instances it is firmly

cemented to its neighboring cells. There is no circulatory system in plants comparable to that of animals to which cells can gain access and within which they can move. The sieve tubes of the phloem are closed at each end by sieve plates. The channels of the xylem are lined with thickened walls through which it is highly improbable that any cell could penetrate. For all these reasons it is improbable that metastases resulting from transport of tumor cells would occur in plants.

The plant also differs from the animal in possessing not single organs but a multiplicity of regions or organs, roots, leaves, stems, growing points, etc. Owing to this multiplicity it is obvious that a tumor in a plant is less likely to prove malignant than it is in an animal. It may destroy one leaf, one stem or one root, but other leaves, stems or roots will compensate for the one destroyed. Plant tumors, therefore, are likely, owing to the nature of the plant's anatomy, to be more restricted and less deadly than are animal tumors. Nevertheless the writers believe that crown-gall tumors do possess the properties which, in animal cancers, "tend to produce death". They possess also the essential characteristic of being able to grow independently of any morphogenetic restraints, upon which all other diagnostic features ultimately depend. The writers conclude, therefore, that "We possess here a plant material which shows all the essential characteristics of a cancerous neoplasm".

It seems, however, that for the discussion of this problem we need a more detailed description of the peculiarities of cancerous cells than is given by the dictionary definition. Such a description has recently been provided by Kidd (62): "Autonomy and anaplasia constitute the distinguishing biological characters of cancer cells, as more than a century of experience has now abundantly shown. The former word can be found in practically every definition of cancer: it means that the cancer cell determines its own activities, largely irrespective of the laws of its host organism; indeed the cells of cancers quite regularly compete harmfully with one another, so heedless of the common good is their proclivity of self-regulation. The word 'anaplasia' is at home largely in the minds and literature of pathologists: it was coined half a century ago by von Hansemann and is used to define the lack of perfect form and function exhibited by the cancer cell in comparison with

its normal fellows". An excessively rapid rate of proliferation is not one of the factors distinguishing the cancer from the normal cell. Many healthy tissues multiply far more rapidly than certain cancers. Kidd adds further, "This brief survey of the characteristics of malignant cells would be incomplete (and unclassical) without pointed reference to the fact that the change to malignancy is permanent, irreversible and hereditary. Cancer cells remain such until they die . . . . They never turn back en masse towards the benign or normal state . . . the individual peculiarities of form and function in the generality of tumor cells are passed on with remarkable fidelity from daughter cell to daughter cell, in many cases during decades, as studies with transplanted cancers have abundantly shown, though the cells of these growths too may sometimes dedifferentiate further and perhaps stepwise in the direction of increasing malignancy".

Crown-gall tumor-tissue as isolated from *Helianthus annuus* or *Vinca rosea* is unquestionably autonomous. Neither in vitro nor when grafted into the intact plant does it show any sign of differentiating or of being influenced by those morphogenic restraints that control the behavior of healthy tissues under such conditions. Anaplasia is also evident in the cells of such crown-gall tissue. Abnormally formed nuclei and multinucleate cells are of common occurrence. Further, it seems permissible to regard these cells as permanently altered, for no evidence has been obtained during the five years which have elapsed since these tissues were first isolated that they have become any less autonomous than they were when first isolated. For this reason White and Braun seem justified in referring to bacteria-free crown-gall tissue of sunflower as a cancerous neoplasm.

The teratomas which can be induced by the crown-gall organism on tobacco, *Bryophyllum* and *Kalanchoë* can not be so easily fitted into the definition of cancer given above. On these plants it would appear that cells which have undergone the transformation induced by the crown-gall organism can change back into normal cells and give rise to organs. The complex tumors such as Braun describes on *Kalanchoë* can not for this reason be referred to as cancerous neoplasms. The cells composing them are neither truly autonomous nor permanently changed, nor can they be described in any sense as malignant. The crown-gall organism has produced

only a temporary change in the growth pattern of these cells comparable to that which can be induced in plant tissues by indole acetic acid and a number of similar compounds.

It seems, therefore, that the crown-gall organism can in certain plants, under certain conditions, transform some cells in such a way that they fit the definition of a cancer cell given above. In other plants it produces a temporary change which does not involve complete autonomy and is reversible. The transformation brought about by the crown-gall organism can not be regarded as an all or nothing effect. The growth patterns of cells exposed to these bacteria are modified in varying degrees, depending on the capacity for growth of the cell itself, the degree of its differentiation, the duration of its exposure to the bacteria and the virulence of the bacteria.

It seems advisable, therefore, to avoid identifying crown-gall with cancer and to describe crown-gall as a disease involving an increase in autonomy of the affected plant tissues which may or may not be reversible. This increase in autonomy of cells is seen in some other plant tumors such as those described by Black (3) on plants infected with the wound tumor virus and those on certain *Nicotiana* hybrids (64, 156). Tissues of the *Nicotiana* hybrid tumors have been found capable of differentiating roots, stems and leaves, and are thus not fully autonomous (157). They correspond in this respect to the tumors described by Braun on *Kalanchoë*. Differentiation of roots has also been observed in cultures of tissues from plants infected with the wound tumor virus (110a).

Phenomena on the molecular level must underly the abnormal growth patterns of all these plant tumors, which, if they could be exposed, might shed light on the mechanisms underlying cancer. The fundamental feature of the cancer cell is not merely that its pattern of growth has been altered but that it transmits the alteration to its progeny. The most important task of the student of cancer is therefore to discover what Kidd has called " the continuing causes of malignancy ". The so-called carcinogenic agents, chemical materials such as methylcholanthrene or physical agents such as X-rays, are clearly not continuing causes. Nor is the crown-gall organism the continuing cause of the abnormal patterns of growth observed in bacteria-free crown-gall tumor tissue. Such

a continuing cause must be sought within the cell itself. It will consist, presumably, of a self-perpetuating alteration of certain constituents, either of the cytoplasm or of the nucleus, the role of which is to regulate the growth and nutrition of the cell. This idea is contained in the hypothesis proffered by Leo Loeb to account for the altered growth pattern of the cancer cell. "Tentatively it may be assumed that . . . a growth substance is produced or increased in quantity and that the production of this substance is renewed autocatalytically. Thus growth processes of certain cells are intensified irreversibly, and certain cellular, metabolic and functional processes are altered . . .". It does not appear, as yet, that students of cancer have succeeded in demonstrating the production of such a growth substance in cancer tissue. There is evidence, however, of the presence of an excess of a growth substance or substances in bacteria-free crown-gall tissue. The lack of response of these tissues to indole acetic acid is possibly due to the presence of an excess of this or a related substance in the tumor tissue. This excess of growth substance may result either from an enhanced capacity on the part of the tumor cell to synthesize the material or from a reduced capacity to destroy it. Rates of growth are influenced by many variables, one of which is the relative rate of synthesis and destruction of essential metabolites. The biochemical mechanisms which regulate these rates in the living plant cell are still obscure, though the studies of Bonner and co-workers (149, 161) on the enzymatic destruction and synthesis of indole acetic acid within the plant promise to shed light on these processes. Probably it is along these lines that the solution of the crown-gall problem can most profitably be sought.

The author is indebted to Dr. A. C. Braun and Dr. Georges Morel for their valuable criticisms of this paper.

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THE PLACE OF SULFUR IN PLANT NUTRITION

FRANK A. GILBERT
Battelle Memorial Institute, Columbus, Ohio

INTRODUCTION

The value of sulfur as a plant nutrient and fertilizer ingredient has been grossly underestimated. Although as a constituent of most proteins it was early shown to be essential to plant growth, the amount of the element needed was not fully appreciated until recently (Miller, E. C., 1938). Undoubtedly this neglect has been detrimental to agriculture in some regions where the deficiency has been severe.

Emphasis in fertilizer practice is laid almost entirely on the three major ingredients—nitrogen, phosphorus and potassium, with calcium next in line. In the literature are many papers on magnesium and iron, and an increasing number concerning the trace elements: copper, manganese, zinc, boron and molybdenum.

But, in proportion to its value, sulfur has been neglected, and if we consider the amount and the number of forms in which it is found in the plant, we can truly say that it is the most underrated plant nutrient.

Sulfur is seldom purchased to supply crop needs. The amounts contributed unintentionally in superphosphate, ammonium sulfate and in mixed fertilizers, together with that obtained gratis from the atmosphere in the form of sulfur dioxide, are sufficient for satisfactory plant growth in some localities. The amount of sulfur incidentally obtained from mixed fertilizers is decreasing with present trends toward higher analysis fertilizers. There has been a tendency to consider that this manna from the air, plus the fertilizer sulfates, is everywhere sufficient and that further applications are superfluous.

This school of thought, under attack for some years, received a severe set-back in the early twenties when soils in the Pacific Northwest were found to respond markedly to applications of sulfur (Powers, 1923). Since that time, sulfur deficiencies have been located in several States. In many others a borderline need was indicated by increased crop yields when sulfur was added to the soil.

The amount of sulfur in a plant is also considerably more than was at one time believed (Fraps, 1900, 1902). Much was lost by the ash-analysis method of determination formerly used. By the more accurate peroxide fusion, or wet ashing methods (Halverson, 1919), it has been found that plants require from two to 100 times as much sulfur as once had been supposed (Thomas et al., 1950).

Sulfur is present in several forms in plant and animal bodies and is a constituent of most protein. The total amount in dry vegetable matter is usually between one-tenth and one per cent. An animal body contains somewhat over one-tenth of one per cent (Corrie, 1948). While plants can make use of sulfates in their nutrition, it is believed that only the sulfur-containing amino acids can be utilized by animals as a source of sulfur.

HISTORICAL

It is impossible to trace the first use of sulfur as a fertilizer, either as gypsum or in other forms. It was used, of course, in organic amendments, such as manures, long before chemists showed that the element was present in these amendments or in any plant or animal material.

According to Alway (1940), the history of sulfur fertilizer may be divided into three periods: the period of gypsum, the period of superphosphate and the modern period. In the first, during the latter half of the eighteenth century and up to about 1850, gypsum was widely used and its beneficial effects were generally overestimated. In the second period, which extended into the first part of the present century, the pendulum swung the other way. Use of superphosphate increased and that of gypsum was discouraged. Although about as much sulfur was used in superphosphate as was formerly used in gypsum, it was assumed to be of minimum importance. The third, or modern period, began with the questioning of this assumption.

The use of gypsum, it is claimed, got its greatest impetus from certain Swiss experiments in 1768 (Crocker, 1920, 1945). Whatever its origin, the use of gypsum spread rapidly and was soon general in western Europe, England and the United States.

The introduction of this material as a fertilizer in the United States dates from a memorable experiment made by Benjamin Franklin. He is said to have chosen a meadow adjacent to a main

road near Washington, D. C., and, according to one version, to have traced in huge letters, with gypsum, the words, " This land has been plastered ". The effect produced on the meadow was a lush growth where the gypsum had been applied, and thus the words could be easily read from the road. Later it was reported that the words could still be seen, but the plants were poorer where the gypsum had been added. The obvious explanation was that heavier crops, stimulated by the gypsum, were taken off the lettered areas. Since no additional fertilizer was added to the field, these areas, now lower in total plant nutrients, made a poorer growth.

The promoters of gypsum fertilization greatly exaggerated its value and importance. They spoke of it as a universal manure, applicable to every variety of soil, for every variety of crop. It is, therefore, understandable why deflation of the gypsum " bubble " was bound to occur. Gypsum was eventually found to produce its best effects on a small group of plants, such as clover and alfalfa, and only on certain soils. In 1840–1844, experiments were carried out which proved the value of the calcium in gypsum and unfortunately thus minimized the value of the sulfur. The worst blow to the planned use of sulfur about this time, however, was the discovery of superphosphate, which ushered in the second period. This material, while providing phosphorus in a readily available form, also furnished even more sulfur which was equally available (Sauchelli, 1950).

In practice, wherever a fertilizer was needed, superphosphate was usually employed—either alone or in combination—frequently with ammonium, potassium or even magnesium sulfates. There was, therefore, a greatly increased but unplanned use of sulfur in fertilizers. Since crops that responded to gypsum did as well or better with superphosphate, the use of gypsum was further discouraged. During this period there appears to have been little or no suggestion that the sulfur content of either gypsum or superphosphate was of any benefit.

Understanding of the true value of sulfur as a plant nutrient and a fertilizer did not come suddenly after the gypsum depression and the discovery of superphosphate. Interest started to pick up at the turn of the century (Bogdanoff, 1899, 1903; Dymond et al., 1905) when the third period began. Even then, although it was

admitted that sulfur was an important essential element, the amount needed was usually underestimated. It was still generally assumed that the supply in the soil was everywhere sufficient.

In 1911 the need for sulfur in the United States was brought into focus by Hart and Peterson in Wisconsin. They demonstrated the needs of crop plants for the element and pointed out to agronomists the advisability of greater consideration of the nutritional value of sulfur to the plant. It was shown that cereal crops remove from the soil about two-thirds as much sulfur as phosphorus, legumes as much or more, and such crops as cabbage, turnips and onions much more sulfur than phosphorus (Crocker, 1923). Anyone who has recognized the sulfide odor of rotting cabbage will not doubt this.

Since publication of the Wisconsin work, similar sulfur surveys have been made for Oregon (Powers, 1930; Reimer and Tartar, 1919), Kentucky (Shedd, 1913), Iowa (Brown and Kellogg, 1915), Kansas (Swanson and Miller, 1917), Indiana (Bertramson et al., 1950) and Vermont (Kelley and Midgley, 1950), and a similar general conclusion was reached. Sulfur-deficient areas have been located in several of the western and midwestern States, and favorable responses to applications of the element have been reported from a number of others over a wide area. General acceptance of the need for sulfur fertilization has been delayed for years, possibly because of the limited areas of acute deficiency.

Today in the eastern United States there appears to be little response to soil applications of gypsum, even on clover and alfalfa fields. This seems peculiar in the light of the reported successful use of this material in the late eighteenth century. There is apparently a greater amount of sulfur in some soils of the eastern United States than was there in the past.

Several reasons for this anomaly have been advanced. The sulfur requirement is supplied in part by manure and fertilizer containing sulfates. Applications of these materials have been greater in the East than in areas now known to be low in sulfur. Industry and urban population are more concentrated in the East. Precipitated sulfur produced by burning coal and other fuels is, therefore, much greater (Alway et al., 1937). An earlier explanation, now discredited by some (Cubbon, 1926; Erdman, 1923;

MacIntire, 1926), is that gypsum increased crop yields by releasing other nutrients, such as potassium, in the soil.

It is probable that soils can be found today in the eastern United States that will give increases in yields of alfalfa and clover with additions of sulfates. Such soils would be those partly exhausted, to which little manure or other sulfur sources have ever been added. They would be located many miles from population centers where little sulfur could be obtained from the air.

LOSSES AND GAINS OF SULFUR IN THE SOIL

The reason for the earlier lack of appreciation of the value of sulfur to plants is not difficult to explain. In the first place, chemists had little idea of the magnitude of the amount of sulfur in a plant. In the ash-analysis method of determination formerly used, only a fraction of the element present was retained, the remainder being lost as volatile material. Secondly, the attitude of many investigators was that the need for sulfur was seldom if ever to be considered in dealing with the fertilizer requirement of agricultural plants. As has already been emphasized, they assumed that precipitated sulfur and that obtained from fertilizers was everywhere sufficient. In the third place, there was a paucity of soils research in regions where there appears to be a question of deficiency in the soil, in the precipitation, or the two together.

The original supply of soil sulfur is fundamentally derived from country rock, but ground water may account for small amounts. Losses of sulfur occur through drainage water, through crop removal, by the burning of crop residues and by release from organic matter in the soil through decomposition. Gains in soil sulfur result from upward leaching, from weathering of parent mineral material, such as various metallic sulfides, from precipitation, especially near industrial centers, and from the application of sulfate-containing fertilizers.

Sulfur is usually classified as a secondary element, but the ratios of sulfur as SO_3 to potassium as K_2O, to phosphorus as P_2O_5, and to nitrogen are, respectively, 0.3 to 0.6, 0.4 and 1.0 in all crops harvested in the United States (Mehring and Bennett, 1950). As has been pointed out, the amount of sulfur removed in crops is nearly equal to that of phosphorus, and in some cases it may be

considerably more. The total loss of sulfur is always much more than that of phosphorus, regardless of the proportionate loss through cropping. This is because of the fact that leaching, erosion and decomposition remove great amounts of sulfur but comparatively little phosphorus.

It has been estimated (Lipman and Conybeare, 1936) that crops remove annually an average of 2.8 pounds of sulfur and 3.8 pounds of phosphorus from each of the 370 million acres of crop land reported in the 1930 census. The annual loss in drainage is over 40 pounds of sulfur but little or no phosphorus (Bizzell, 1926; Lyon and Bizzell, 1916). Addition of phosphorus in fertilizers to the same 370 million acres was estimated at 3.5 pounds per acre and that of sulfur at 3.9 pounds, thus leaving a deficit of approximately 0.3 pound of phosphorus per acre as compared with a net approximate loss of 28.6 pounds of sulfur—95 times as much. In computing the average net loss of sulfur, the 14.6 million acres of irrigated crop land was omitted. This land receives an estimated annual addition from irrigation water of over 200 pounds of sulfur per acre.

The difference of 16 pounds per acre between the gross and net loss of sulfur is accounted for by 1.5 pounds in manure, crop residues and seeds, 3.9 pounds in commercial fertilizers, and 10.6 pounds from rainfall. Alway (1940) considers the estimate of 10.6 pounds from rainfall to be too high. Similar estimates of the comparative losses of sulfur and phosphorus have been made by other investigators and the same general conclusions were reached.

Certain soils that have been cropped for a generation appear to have lost 20% to 40% of their initial sulfur content (Shedd, 1914). Woodward, in 1922, estimated that the highest sulfur content of the soils examined by him was sufficient for 39 years of alfalfa, 355 years of wheat and 232 years of corn, while the lowest sulfur content was sufficient for only five years of alfalfa, 46 years of wheat and 30 years of corn.

Several of the more important fertilizer materials, such as superphosphate and ammonium sulfate, contain more SO_3 than N, P_2O_5 and K_2O combined (Mehring and Bennett, 1950). The amounts of sulfur added in this way tend to decrease with increasing use of higher analysis fertilizers. Fungicides, insecticides and

animal manures also supply sulfur, but the amount is small compared to that in fertilizers and that obtained from the air. The SO_3 contents of virtually all materials used as fertilizers, manures and soil amendments have been published (Mehring and Lundstrom, 1938).

Determinations of the amount of sulfur in rainfall have been carried out for at least 100 years. In all cases in Europe, England and the United States it was found that the sulfur content of the air increases rapidly in the vicinity of cities and large towns because of the sulfur in the coal used.

In Minnesota the annual fall of sulfur in rain, snow and dust varies from a maximum of around 100 pounds per acre in urban areas to a minimum of less than five pounds per acre in rural areas (Alway et al., 1937). The largest sulfur precipitation ever reported was 232 pounds per acre per year near a smelter in Tennessee (MacIntire and Young, 1923). In general, 15 to 30 pounds are deposited in rural areas (Erdman, 1922; Johnson, 1924). Plants may contain several times as much sulfur in localities where there is a distinct contamination from factories as they will contain in regions not subject to this contamination (Frazer, 1935).

In all cases, reports of sulfur from rain will tend to be too high rather than too low because of the many sources of error in the collection of samples. The concentration of sulfur in rain water will increase after being poured from a rain gauge or other collecting vessel and will steadily increase with continued exposure to the atmosphere. Sulfur dioxide is the disturbing agent. It attacks all metal surfaces, and if the metal gauges are not protected by a sulfur dioxide-resistant coating, sulfates will be formed and carried down in the next rain. Cotton fabric coated with lead peroxide paste exposed to the air but protected by a roof will determine the amount of sulfur in the air more accurately than analysis of rain.

There are many reports of increased crop yields resulting from application of sulfur, even though there appeared to be a sufficient amount in the soil (Cross, 1926; Duley, 1916; McKibben, 1928; Roux, 1928). This may be attributed partly to the beneficial effect of increased acidity instead of an increase in water-soluble sulfates. Exchangeable cations are replaced by hydrogen, thereby

increasing the amount of water-soluble cations (Aldrich and Turrell, 1950). The solubility of soil phosphates may be increased or decreased, depending on the amount of calcium that is released (Chapman, 1936; Ligon, 1935; Lipman, 1924; Lipman et al., 1916). The solubility of metallic ions, such as copper, iron, manganese and zinc, is increased. The beneficial effect may be temporary, since acidification if combined with heavy leaching means rapid loss of nutrients.

LOCATIONS OF SULFUR DEFICIENCY IN NORTH AMERICA

Recognized sulfur deficiencies in crops are usually associated with early stages of growth under dry conditions. Several regions have been found in which the supply of available sulfur in the soil is insufficient to give maximum yields, and addition of some form of the element in those regions increases crop growth.

Sulfur fertilizer in the elemental form is believed to have been first tested at Corvallis and Redmond, Oregon, in 1912. The trials were extended and eventually showed that the soils of the State were generally low in sulfur (Powers, 1923; Reimer and Tartar, 1919) and that some 80% of the alfalfa land would respond to the element. In some parts of the State the wide disparity between sulfur and phosphorus is increasing, making sulfur fertilization even more imperative (Jones, 1928).

Sulfur-deficient areas have been reported also from other States, including Washington (Olson and St. John, 1921), California (Chapman and Brown, 1941), Idaho (Neidig et al., 1923), Montana (Burke and Morris, 1941), North Dakota (Painter, 1943), Florida (Harris et al., 1945), Arkansas (Younge, 1938, 1942) and Minnesota (Alway, 1927, 1940) as well as the Canadian provinces of Alberta (Newton, 1936), Saskatchewan (Alway, 1927) and Ontario (Alway, 1927). Eaton (1922) reported that certain soils from Alabama, Maryland and Oklahoma were deficient in sulfur. Reports from other States (Ames and Blotz, 1913; Bertramson and Fried, 1949; Bollen, 1925; Fraps, 1930; Greaves and Gardner, 1929; Hart and Tottingham, 1915; McGeorge, 1945; Shedd, 1917; Taubenhaus and Roney, 1934; Volk et al., 1945) on the value of sulfur fertilization may indicate that deficiencies occur in these States also.

Remarkable results have been obtained by adding sulfur to the soil. Yields of alfalfa were increased as much as 500 to 1000 per

cent on some Oregon soils (Bradley, 1910; Brown, 1917). Sugar beets also grew better (Tolman and Stoker, 1941). In other States increased yields of alfalfa and other legumes were obtained (Ames, 1923; Bledsoe and Blaser, 1947; Bollen, 1925; Duley, 1916; Evans and Greaves, 1937; Harston et al., 1944; St. John and Neller, 1924; Tottingham, 1918).

Early studies at the Illinois Agricultural Experiment Station indicated that, in general, there would be no need to add sulfur to Illinois soils in a permanent system of soil fertility (Stewart, 1920). However, a later bulletin from the same station reported a wide variation in the sulfur content of Illinois soils and stated that in the southern part of the State there were soils quite deficient in sulfur.

A disease of Burley tobacco due to sulfur deficiency has been reported (Valleau, 1935; Valleau and Johnson, 1936) in Kentucky. In California there is an increasing number of cases where soils do not respond to applications of lime, phosphorus or potassium but where excellent results are obtained by use of sulfur or gypsum (Conrad, 1950).

Cultivated soils in any region where the precipitation of sulfur from the air is less than ten pounds per acre are in danger of sulfur shortage. Sulfur deficiency is almost always accompanied by deficiencies of one or more of the primary elements and in many cases has been masked by them.

SULFUR IN PLANTS

Sulfur occurs in plants chiefly in proteins, volatile compounds and sulfates. The amount varies considerably in different families and genera of plants, and in some cases the plant contains more sulfur than calcium, phosphorus or magnesium. Many brassicaceous[1] plants, for example, are so rich in the element that they may be considered as sulfur carriers. The strong odors generated in cooking such plants, or in their decay, are largely due to their high sulfur content.

The sulfur of protein is centered in the amino acids, cystine and methionine, found in all plants. The proportionate amounts of cystine and methionine have been increased in alfalfa, along with a general increase in the protein content, by sulfur fertilization (Hauge et al., 1949; Tisdale et al., 1950). The sulfur-bearing

[1] Members of the cabbage tribe of the mustard family.

vitamins, thiamine and biotin, are also essential to plant metabolism.

In the volatile form, sulfur occurs in mustard oil, allyl and vinyl sulfides, and the mercaptans (Peterson, 1914). Most of these volatile compounds are limited to certain plant families, and little is known of their significance to the plants that synthesize them or to the animals that consume them. The mustard oils do not occur as such in the plant but are in the form of glucosides which hydrolyze to mustard oil. The Tropaeolaceae, and the onion tribe of the Liliaceae, form mercaptans and allyl and vinyl sulfides. Members of the Resedaceae, Capparidaceae, Umbelliferae and other plant families synthesize special organic sulfur compounds.

Sulfur occurs in the inorganic form in plants, principally as sulfates. As much as 65% of the sulfur found in certain plants is in this form. Plants cannot use elemental sulfur. Apparently it must be oxidized to the sulfate form before it is of any benefit to them. All of the sulfates produce similar beneficial results, insofar as sulfur is concerned, when applied as fertilizer (Reimer and Tartar, 1919).

Sulfur is fairly evenly distributed throughout the plant. In a mature corn plant approximately 40% of the total amount is in the leaves, 23% in the stem, 26% in the grain, and 11% in the roots (Peterson, 1914). Older tissues tend to have a higher sulfur content, as evidenced by yellowing of the younger leaves when a plant first becomes sulfur deficient.

Sulfur produces certain formative effects in plants in addition to its function as a building material. Among these are an effect on the chlorophyll, an increased root system (Miller, H. G., 1919; Pitz, 1916) and an effect on the nodules of legumes (Duley, 1916; Miller, H. G., 1921).

Plants grown on a sulfur-deficient soil have a pale green color. Application of sulfur to the soil, or dusting with sulfur or gypsum, will change the leaf color to a dark healthy green (Eaton, 1922) and increase the chlorophyll content as much as 18% (Powers, 1930). The exact physiological effect of the sulfur is not known, but it appears to be indirect, since chlorophyll does not contain sulfur.

The effect of sulfur fertilizer on the root system has been noted many times. The roots of sulfur-treated plants may be several

times as large as those from unfertilized plants. It has been suggested (Crocker, 1923) that the increased root development often observed when superphosphate is used is due in part to the sulfates in the superphosphate rather than entirely to the phosphorus.

Plants having a high protein content respond most markedly to application of sulfur as a fertilizer. In the literature there are many examples of the beneficial effect of sulfur on the production of legumes. In most cases, not only is the growth stimulated, but the protein content is increased (Brown, 1917; Miller, H. G., 1919; Neller, 1926). A deficiency of sulfur in legumes results in limited synthesis of protein and restricted growth (Tottingham, 1918).

Nodule development on the roots of legumes is promoted by use of sulfur (Duley, 1916; Miller, H. G., 1921), since legumes growing on a soil containing sufficient sulfur show a larger number of nodules than legumes from a deficient soil. The better nodule development which stimulates the growth of legumes has been attributed to the action of sulfates on the legume bacteria (Powers, 1923). Legumes fertilized with sulfates have shown a higher nitrogen content than unfertilized controls, while fertilized rape plants which assimilated a large amount of sulfur did not show any increased nitrogen content over the controls.

In Utah (Fife, 1926) it was found that, in soils containing considerable organic matter, sulfur increased the bacterial numbers, but in soils deficient in organic matter the bacterial numbers decreased as the sulfur increased. From South Carolina it has been reported that the sulfur content of tobacco leaf is influenced by the sulfur in the fertilizer (Albert and Lunn, 1935). Use of high-sulfur superphosphate increased the sulfur content of the leaves as much as 48% over that resulting from use of a low-sulfur triple superphosphate. There is a measurable decrease in the general sulfur content of vegetation with increasing distances from urban and industrial districts (Katz and McCallum, 1939; McCool and Johnson, 1938).

SYMPTOMS OF SULFUR DEFICIENCY

The effects of sulfur deficiency in many ways resemble those caused by a deficiency of nitrogen. This is probably due to the connection of sulfur with the formation of proteins and chlorophyll.

The deficiency is characterized by loss of green color in the younger leaves of the plant, including the veins. When the deficiency is severe, all the leaves are light green, but the lower leaves do not dry up as they do in the case of insufficient nitrogen.

Sulfur-deficient plants are higher in nitrogen, soluble sulfur compounds and starch, and lower in sugars than plants with sufficient sulfur (Eaton, 1941). The accumulation of nitrates and carbohydrates seems to be due to low nitrate assimilation resulting from low reductase activity. Such plants have thick cell walls and a relatively high proportion of fibers and lignified tissue. The protoplasm appears much like that of plants lacking nitrogen in that it is limited in amount but is not noticeably injured, as it is when phosphorus, calcium or potassium is deficient. Ergle and Eaton (1951) have compared in tabular form the chemical and physical characteristics of cotton and other species of plants when subjected to low levels of sulfur nutrition.

Sulfur deficiency in the navel orange (Chapman and Brown, 1941) is characterized by an abnormal yellowing of the new-cycle growth. The bloom is profuse but weak. Sulfur-deficient fruits are light yellowish green and on maturing fail to turn to a deep orange color. Most of the sulfur-deficient fruits show reduced juice content and abnormally thick rinds. The juice vesicles frequently are shriveled.

The only named deficiency disease attributed with certainty to a lack of sulfur is the " tea yellows " of Nyasaland (Forbes, 1942; Storey and Leach, 1933). In recent years this physiogenic disease has seriously handicapped tea production in that country. The disease is characterized by restricted shoot growth and dwarfed leaves which become stiff, brittle and chlorotic. The terminal buds and small branches die back. Any type of organic or inorganic fertilizer which contains sulfur in an available form will cure " yellows ". Observation showed that tea bushes can not maintain a reserve of sulfur internally. Sulfur must always be available to the roots.

SULFUR IN ANIMALS

Green plants, because of their synthetic power, are the primary source of sulfur for animals which are unable to form proteins from the inorganic sulfates (Daniels and Rich, 1918). Only a

very small amount of sulfur is found in the animal body in the form of sulfates. Practically all of it occurs in proteins containing the amino acids cystine and methionine. Cystine was regarded as the "essential" amino acid until the discovery of methionine. While both are important, animals will die without methionine, even though an abundance of cystine is provided. On the other hand, it has been demonstrated that cystine may be omitted from the diet if methionine is present.

Cystine is the principal sulfur constituent of keratins, such as those of hoof, horn, hair and feathers. This amino acid gives rise to cysteine and probably to taurine, a constituent of one of the bile acids. Methionine is almost completely absent from protein derived from skin sources. Wool contains 13% of cystine and about 4% of sulfur. Human hair, especially red hair, contains over 5% of sulfur.

Biotin and vitamin B_1, or thiamine, are biologically important sulfur-containing compounds, as is insulin, in which the sulfur is in the form of cystine (Forman, 1943). Thiocyanates, also containing sulfur, have been found in the saliva and other body fluids. It is believed that they may be the result of the conversion of poisonous cyanides to a relatively harmless form. Other organic forms of sulfur found in the body are glutathione, ergothioneine, taurocholic acid, "ethereal sulfates" (esters of phenols and sulfuric acid), chondroitin sulfuric acid (in cartilage) and melanins (Kahn and Goodridge, 1926).

The body can reutilize the sulfur-containing amino acids. It has been estimated that 30% of the cystine produced in the body from the breakdown of protein is again utilized in the liver (Wirtschafter, 1942). The amount of sulfur excreted daily depends on the sulfur intake, which in turn depends on the amount of protein that is eaten or metabolized.

Sulfur is not ordinarily considered in connection with problems of mineral deficiency in animals because if the diet contains sufficient protein of the right kind, this element will be furnished in adequate amounts. If the animal or human diet should be deficient in sulfur, that element would be directly connected with the more inclusive problem of protein deficiency. In North Dakota, where pregnancy disease in ewes was prevalent, the sulfur content of grasses and oat straw and, incidentally, the protein content of

this material, was found to be very low, and it was doubtful that farm animals could obtain sufficient sulfur-containing amino acids in their diet.

Many conflicting opinions exist concerning the use of sulfur in the treatment of various pathological conditions. There is no evidence that inorganic sulfur introduced into the body in any way can be used for the synthesis of sulfur-containing amino acids which alone can be utilized by man as a source of this element (Daniels and Rich, 1918).

Inorganic sulfur was once a common ingredient of mineral supplements for farm animals and even today is found in some, in spite of evidence that inorganic sulfur compounds have no nutritive value. On the other hand, there is plenty of evidence that excessive sulfur in the elemental form is toxic to animals. The sulfur and molasses treatment formerly administered each spring by the older generations to the younger was, needless to say, greatly overrated.

SULFUR AS A SOIL CORRECTIVE

ALKALINE SOILS. In calcareous or alkaline soils many crops are unable to properly utilize micronutrient elements, such as iron, manganese, copper, zinc and boron, although these soils are seldom actually deficient in the elements. In extreme cases accumulation of basic salts makes agriculture on such soils impossible or at least unprofitable.

When there is an actual or physiological deficiency of one or more micronutrient elements, it is usually manifest by some type of chlorosis or loss of green coloring matter in the leaves. In severe cases the chlorosis is accompanied by reduced leaf size, stunted growth, dieback and general decline. One such trouble is known as "limestone chlorosis" because it is found on soils containing an excess of lime. Since such soils are widely distributed over the world, the trouble is not uncommon.

Some years ago the University of Kentucky had difficulty in establishing newly planted pin oaks on its campus. The trouble was remedied after it was realized that the high pH of the subsoil was responsible. In Columbus, Ohio, the soils to the west of the city are derived from Devonian limestone, those to the east from Devonian shales. Chlorosis on pin oaks is common on the limestone but has not been noticed on the shale. Limestone spread

on roads in the Florida Everglades has caused a manganese deficiency in the fields adjacent to the roads. The blowing and washing of the limestone raised the soil pH high enough to tie up the manganese that was present.

Agriculturalists have turned to sulfur as the most satisfactory material for solving the problem of limestone chlorosis. At the Arizona experiment station, sulfur has been used both alone and in combination with manure to correct citrus chlorosis. For deficiencies in individual trees or shrubs, sulfur is placed in soil auger holes around the drip of the tree or in furrows. The amount to apply varies with the age of the tree, the pH of the soil and the severity of the chlorosis.

In many parts of the West reclamation of alkaline land is one of the most difficult soil problems (Fraps, 1930). The alkaline soils are of two types, white and black. In white alkali soils an excess of soluble mineral salts may be injurious to plant growth, but the soil is usually of mellow structure. Black alkali soils have a high proportion of sodium salts which react with water to form sodium hydroxide. The excess hydroxide makes the soil so impermeable that it will not take water readily, and seedling growth is hampered because it is difficult for the plant to absorb water from such a soil.

Application of sulfur is the best known single treatment for alkaline soils of either type. The sulfur, largely as a result of bacterial action (Starkey, 1950), oxidizes to sulfuric acid which reacts with calcium carbonate to release soluble calcium. This is carried off in the drainage water. In black alkali soils the calcium displaces sodium during the leaching operation and at the same time flocculates the soil sufficiently to increase the rate of water movement downward. White alkali is neutralized black alkali. If the soil can not be drained, or if there is no free circulation of air and moisture for sulfur oxidation, there can be no reclamation.

Obviously application of sulfur to white alkali lands should be at a lighter rate than for black alkali lands. A conservative initial application is 200 to 400 pounds per acre for white alkali soil and from 1000 to 2000 pounds per acre for black alkali soil. For maintaining good soil structure on these lands, 100 to 200 pounds per acre annually is recommended (McGeorge, 1945).

In the East conditions are quite different. The salts of calcium and other elements have been leached out to a large extent over a

period of centuries. The excessive amount of sulfur that enters the soil near cities and industrial operations oxidizes to sulfuric acid and greatly accelerates the natural leaching of nutritive elements. Here soils in many places are too acid instead of being too alkaline, and it is desirable to add lime in lieu of leaching this material away in the drainage.

SELENIFEROUS SOILS. In certain of the Great Plains States the selenium content of some soils creates an agricultural problem. Selenium is toxic to animals but is tolerated within limits by most plants. Many plants are able to accumulate the element in large amounts. The problem lies in finding some way of making the vegetation growing on these soils safe for animal consumption.

The sulfur content of the nutrient medium influences both the uptake of selenium by plants and its toxicity to the plant. Data indicate that an increase in the amount of available sulfur tends to inhibit the accumulation of selenium by the plant and to reduce the injurious effect of that which is absorbed (Kiplinger and Fuller, 1946). Except possibly for greenhouse soils and special cases, sulfur-selenium antagonism is not likely to provide a practical means of preventing crop plants from accumulating toxic concentrations of selenium, since field tests have not entirely corroborated laboratory findings (Trelease, 1945).

CONTROL OF SOILBORNE DISEASES BY SULFUR. Sulfur and its compounds constitute one of the most effective and commonly used types of fungicide. Its secondary value in the control of soil organisms is less important and less well known.

Potato scab is one of the best known diseases whose incidence depends largely on the pH of the soil. A high pH favors the occurrence of this and some other bacterial diseases. Since application of sulfur is one of the most common and effective methods of lowering soil pH, it is indirectly effective in controlling such disease. Experiments have also shown that applications of 300 to 400 pounds of sulfur per acre will reduce loss from sweet potato scurf and soil rot on some soils (Cook, 1931).

SUMMARY

Sulfur is necessary to both plant and animal life. The amount needed by plants, however, has been underestimated until recently.

The amount of sulfur removed in crops is nearly equal to that

of phosphorus, but the total loss is more because much sulfur but little phosphorus is lost through leaching. In urban and industrial areas this loss is not serious because considerable sulfur accrues through deposition from the air. Localities with soil under cultivation, where the sulfur precipitation amounts to less than ten pounds per acre per year, are in danger of sulfur shortage.

Sulfur-deficient areas have been reported from several of the western States, and there is evidence that some locations in the Midwest would profit from sulfur applications also.

Sulfur occurs in plants in certain proteins, volatile compounds and sulfates. It is believed to stimulate root growth and to have a beneficial effect on legume bacteria. Sulfur deficiency is characterized by reduced growth, chlorosis and twig dieback. The "yellows" disease of tea is caused by a lack of sulfur in the soil.

In alkaline soils many crops are unable to properly utilize micro-nutrients, and in severe cases fail to make any growth. On such soils sulfur changes the alkali to a soluble form in which it can be washed away.

Sulfur is an essential for animal life also. In animals it is chiefly found as part of the amino acids, cystine and methionine, of the protein. It is also contained in certain vitamins and other organic compounds.

Sulfur is not ordinarily thought of in connection with problems of mineral deficiency of animals because the diet, if it contains sufficient protein, it will contain sufficient sulphur.

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